Guidance for Industry

Bioanalytical Methods Validation for Human Studies

DRAFT GUIDANCE

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Draft — Not for Implementation

GUIDANCE FOR INDUSTRY[1]

Bioanalytical Methods Validation for Human Studies

I. INTRODUCTION

This guidance provides assistance to sponsors and applicants of investigational new drug applications (INDs), new drug applications (NDAs), abbreviated new drug applications (ANDAs), and supplements, in developing validation information for bioanalytical methods used in human clinical pharmacology, bioavailability (BA), and bioequivalence (BE) studies. The guidance does not address analytical methods used for nonhuman pharmacology/toxicology studies, CMC information, or in vitro dissolution studies.

The information in this guidance is generally applicable to gas chromatography or high-pressure liquid chromatography analytical methods performed on drugs and metabolites obtained from biological matrices such as blood, serum, plasma, or urine. This guidance should also apply to other analytical techniques such as immunological and microbiological methods or other biological matrices, such as tissue samples including skin samples, although in these cases a higher degree of variability may be observed.

II. BACKGROUND

This guidance is based primarily on a conference on Analytical Methods Validation: Bioavailability, Bioequivalence and Pharmacokinetic Studies, which was held on December 3 - 5, 1990, and sponsored by the American Association of Pharmaceutical Scientists, U.S. Food and Drug Administration, Federation Internationale Pharmaceutique, the Canadian Health Protection Branch, and the Association of Official Analytical Chemists (Shah 1992).

Selective and sensitive analytical methods for the quantitative determination of drugs and their metabolites (analytes) are critical for successful performance of clinical pharmacology, BA, and BE studies. Analytical method validation includes all of the procedures recommended to

[1] This guidance has been prepared by the Biopharmaceutics Coordinating Committee and the Clinical Pharmacology Section of the Medical Policy Coordinating Committee in the Center for Drug Evaluation and Research (CDER) at the Food and Drug Administration. This guidance document represents the Agency's current thinking on validation of analytical methods for human studies based on drug or metabolite assay in a biological matrix. It does not create or confer any rights for or on any person and does not operate to bind FDA or the public. An alternative approach may be used if such approach satisfies the requirements of the applicable statute, regulations, or both.

demonstrate that a particular method for the quantitative measurement of an analyte in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible. The parameters essential to this validation include (1) accuracy, (2) precision, (3) sensitivity, (4) specificity, (5) linearity, and (6) reproducibility. In addition, the stability of the analyte in the matrix under study storage conditions should be determined. Validation involves documenting through the use of specific laboratory investigations that the performance characteristics of the method are suitable and reliable for the intended analytical applications (Shah 1992, Taylor 1983). The acceptability of analytical data corresponds directly to the criteria used to validate the method.

Published methods of analyte analysis are often modified to suit the requirements of the laboratory performing the assay. These modifications should be validated to ensure suitable performance of the analytical method. When changes are made to a previously validated method, the analyst should exercise judgment as to how much additional validation is needed. For minor modifications, such as a change in the ratio of solvents for elution, a change in buffer system, the number of extractions of the biological matrix, or a small change in column temperature to obtain better separation, only limited validation may be recommended. For major modifications, such as change of an instrument, solvent system, detector, or temperature, full validation of the modified method should be performed.

The analytical laboratory conducting BA and BE studies should closely adhere to FDA's Good Laboratory Practices (GLPs) (21 CFR Part 58) and to sound principles of quality assurance throughout the testing process. In addition, the analytical methods for in vivo bioavailability studies must meet the criteria in 21 CFR 320.29. The analytical laboratory should have a written set of standard operating procedures (SOPs) to ensure a complete system of quality assurance. The SOPs should cover all aspects of analysis from the time the sample is collected and reaches the laboratory until the results of analysis are reported. They also should include record keeping, security and chain of sample custody (accountability systems that ensure integrity of test articles), sample preparation, and analytical tools, such as methods, reagents, equipment, instrumentation, and procedures for quality control and verification of results.

The process by which a specific analytical method is validated may be divided into (1) reference standard preparation, (2) pre-study validation for analytical method development and method establishment, and (3) in-study validation to include study performance, drug analysis, and acceptance criteria (Shah 1992, Brooks 1985). These three processes are described in the following sections of the guidance.

III. REFERENCE STANDARD

Analysis of drugs and their metabolites in a biological matrix is invariably carried out using samples spiked with calibration standards and quality control (QC) samples. The quality of the reference standard used to prepare spiked samples can affect study data. For this reason, an authenticated analytical reference standard should be used to prepare solutions of known concentrations. If possible, the reference standard should be identical to the analyte. When this is not possible, an established chemical form (free base or acid, salt or ester) of known purity can be used as a surrogate. Three types of reference standards are usually used: (1) certified reference standards (e.g., USP compendial standards); (2) commercially supplied reference standards obtained from a reputable commercial source; and/or (3) other materials of documented purity custom-synthesized by an analytical laboratory or other noncommercial establishment. The source and lot number, certificates of analyses when available, and/or internally or externally generated evidence of identity and purity should be furnished for each reference standard. A ***master standard*** (a synthetic batch for which identity and purity are clearly established and acceptable) should be maintained for each reference standard. All subsequently synthesized batches are to be compared chromatographically with that master standard. All reference materials should be checked prior to use to determine if there are significant interfering chromatographic peaks at the retention time of the analyte and/or the internal standard, using the analytical procedure to be used in the study.

IV. PRE-STUDY VALIDATION

Pre-study validation should include analytical method development and documentation. Validation should be performed for each biological matrix and for each chemical species to be measured in the biological matrix (Shah 1992, Buick 1990). In addition, the stability of quality control samples and the analyte in spiked samples should be determined. Typical performance parameters that should be assessed during pre-study validation include (1) specificity, (2) calibration curve and its linearity, (3) precision, accuracy, recovery, (4) quality control samples, (5) stability of analyte in spiked samples, and (6) acceptance criteria.

A. Specificity

Specificity is the ability of an analytical method to differentiate and quantitate the analyte in the presence of other constituents in the sample and refers directly to the ability of the method to produce a response for a single analyte (Karnes 1991). For specificity, analyses of blank samples of the appropriate biological matrix (plasma, urine, or other matrix) should be obtained from six individuals under controlled conditions, with reference to time of day, food ingestion, and other factors considered important in the intended study. Each blank sample should be tested for interference using the proposed extraction procedure and chromatographic or spectroscopic conditions. The results should be compared to

those obtained with an aqueous solution of the analyte at a concentration near the limit of quantitation (LOQ).

Any blank sample with significant interference at the retention time of the drug, metabolites, or internal standard should be rejected. If more than 10% of the blank samples exhibit significant interference at these retention times, additional matrix blank samples should be tested. If more than 10% of this subsequent group of blank samples still shows interference, the method should be changed to eliminate the interference.

Potential interfering substances in a biological matrix include endogenous matrix components, metabolites, decomposition products, and, in the actual study, concomitant medication. Potential interference from nicotine and common OTC drugs and metabolites, such as caffeine, aspirin, acetaminophen, and ibuprofen should be routinely tested. If the method is intended to quantitate more than one analyte, each analyte should be injected separately to determine its retention time and to ensure that impurities from one analyte do not have the same retention time as another analyte.

B. Calibration Curve

Calibration is the relationship between instrument response and known concentrations of the analyte. A calibration (standard) curve should be generated for each analyte in the sample. A sufficient number of standards should be employed to adequately define the relationship between concentration and response. A calibration curve should be prepared in the same biological matrix as the samples in the intended study by spiking with known concentrations of the analyte. Precautions should be taken to avoid precipitation while spiking the biological matrix. The number of standards used in constructing a calibration curve will be a function of the anticipated range of analytical values and the nature of the analyte/response relationship. Concentrations of standards should be chosen on the basis of the concentration range expected in a particular study. A calibration curve should consist of a blank sample (matrix sample processed without internal standard), a zero sample (matrix sample processed with internal standard), and five to eight non-zero samples covering the expected range, including lower LOQ. Blank and standard zero samples should not be used in the calibration function, but should only serve to evaluate interference. Additional factors in developing a calibration curve relate to LOQ and linearity.

1. Limit of Quantitation (LOQ)

The lowest standard on the calibration curve should be accepted as the limit of quantitation if the following conditions are met:

- No interference present in blanks at the retention time of the analyte at this concentration, or typical response at this concentration at least five times greater than any interference in blanks at the retention time of the analyte

- Analyte peak (response) identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120% (Shah 1992).

2. Linearity

The simplest workable regression equation should be used with minimal or no weighting. Selection of weighting and use of a complex regression equation should be justified. Four factors should be met in developing a calibration curve:

- ≤20% deviation of the LOQ from nominal concentration (Shah 1992)

- ≤15% deviation of standards other than LOQ from nominal concentration (Shah 1992)

- At least four out of six non-zero standards meeting the above criteria, including the LOQ and the calibration standard at the highest concentration

- 0.95 or greater correlation coefficient (r)

C. Precision, Accuracy, and Recovery

The ***precision*** of an analytical method describes the closeness of individual measures of an analyte when the procedure is applied repeatedly to multiple aliquots of a single homogeneous volume of biological matrix. Precision should be measured using a minimum of five determinations per concentration. A minimum of three concentrations in the range of expected concentrations is recommended. The precision determined at each concentration level should not exceed 15% coefficient of variation (CV) except for the LOQ where it should not exceed 20% CV. Precision is further subdivided into within-day, intra-batch precision or reproducibility, which assesses precision during a single analytical run, and between-day, inter-batch precision or reproducibility, which measures precision with time and may involve different analysts, equipment, reagents, and laboratories (Shah 1992, USP XXII 1990, Brooks 1985).

The ***accuracy*** of an analytical method describes the closeness of test results obtained by the method to the true value of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. A minimum of five determinations

per concentration should be conducted for a minimum of three concentrations in the range of expected concentrations. The mean value should be within 15% of the actual value except at LOQ, where it should not deviate by more than 20%. The deviation from the true value serves as the measure of accuracy (USP XXII 1990, Brooks 1985).

The ***recovery*** of an analyte in an assay is the detector response obtained from an amount of the analyte added to and recovered from the biological matrix, compared to the detector response obtained for the pure authentic standard (Brooks 1985, Mehta 1989). Recovery pertains to the extraction efficiency of an analytical method within the limits of variability. Although recoveries close to 100% are desirable, the extent of recovery of an analyte and/or the internal standard may be as low as 50 to 60% if the recovery is precise, accurate, and reproducible. Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations (low, medium, and high) with unextracted standards that represent 100% recovery.

D. Quality Control Samples

Pre-study validation of an analytical method should be carried out using at least three batches of biological matrix, where each batch is collected from a different source. Each batch should contain (1) a calibration curve constructed using a blank sample, zero sample, and five to eight non-zero standards, (2) LOQ quality control (QC) samples, (3) low QC samples, (4) medium QC samples, (5) high QC samples, (6) a matrix blank sample, and (7) a reference standard. Quality control samples at concentrations noted below should be made from a stock solution separate from that used to prepare the standards.

***LOQ QC sample*:** Same concentration as the lowest non-zero standard

***Low QC sample*:** $\leq$3 x LOQ

***Medium QC sample*:** Approximately midway between the high and low QC concentrations

***High QC sample*:** 75 to 90% of highest calibration standard

The accuracy of preparation of calibration and QC samples should be checked with the first batch. The data from replicate analyses of QC samples and duplicate analyses of reference standards should be used to obtain the intra-day (within batch) precision, inter-day (between batch) precision, accuracy, and recovery.

To obtain within-batch data, the mean, standard deviation, and CV of each QC concentration in each batch should be calculated. The global (overall) mean, standard deviation, and CV for each QC concentration from the three batches should be calculated to obtain between-batch data. Precision is indicated by the %CVs. Percent accuracy is determined by dividing the mean concentration of a QC by its nominal concentration, and multiplying by 100.

E. Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. The stability of an analyte in a particular matrix and container system is relevant only to that matrix and container system and should not be extrapolated to other matrices and container systems. Stability procedures should evaluate the stability of the analytes in biological fluids after long-term (frozen at the intended storage temperature and conditions) and short- term (bench top, room temperature and conditions) storage, and after going through freeze and thaw cycles and the analytical process. The procedure should also include an evaluation of analyte stability in stock solution (Buick 1985, Pachla 1989).

All stability determinations should use a set of standard samples prepared from a freshly made stock solution of the analyte in the appropriate analyte-free, interference-free biological matrix. Stock solutions of the analyte for stability evaluation should be prepared in an appropriate solvent at concentrations defined in the method SOP. Further information about validation for these factors appears in the following five sections of the guidance.

1. Freeze and Thaw Stability

Testing for freeze and thaw analyte stability should be determined during three freeze and thaw cycles. At least three aliquots at each of the low and high concentrations should be stored at -20^{0}C, or the intended storage temperature, for 24 hours and thawed unassisted at room temperature. When completely thawed, the samples should be transferred back to the original freezer and kept refrozen for 12 to 24 hours. The cycle of thawing and freezing should be repeated two more times, then analyzed on the third cycle. If an analyte is unstable at -20^{0}C, the stability sample should be frozen at -70^{0}C during the three freeze and thaw cycles (Shah 1992, Buick 1990).

2. Short-Term Room Temperature Stability

Three aliquots of each of the low and high concentrations should be thawed at room temperature and kept at this temperature from 4 to 24 hours (based on the expected duration that samples will be maintained at room temperature in the intended study) and analyzed (Buick 1990).

3. Long-Term Stability

The storage time in long-term stability evaluation should exceed the time between the date of first sample collection and the date of last sample analysis. Long-term stability should be determined by storing at least three aliquots of each of the low and high concentrations under the same conditions as the study samples. A suggested storage temperature for the majority of drugs and metabolites in a biological matrix is -20^0C, but lower temperatures (e.g., -70^0C) may be recommended to prevent degradation problems observed at higher temperatures. The volume of samples should be sufficient for analysis on three occasions. The concentrations of all the stability samples should be compared to the mean of back calculated values for the standards at the appropriate concentrations from the first day of long-term stability testing (Buick 1990).

4. Stock Solution Stability

The stability of stock solutions of drug and the internal standard should be evaluated at room temperature for at least 6 hours. The stability samples should then be refrigerated or frozen for 7 to 14 days or other relevant period. After completion of the desired storage time, the stability should be tested by comparing the instrument response with that of freshly prepared solutions (Buick 1990).

5. Autosampler Stability

The stability of processed samples in the autosampler should be determined at the autosampler temperature that will be used during analysis, which is usually room temperature, but may sometimes be a lower temperature (e.g., when a refrigerated autosampler is used). Stability should be assessed over the anticipated run time for the batch size to be used in studies. The stability of both the drug and the internal standard should be evaluated in validation samples under these conditions by determining concentrations on the basis of original calibration standards.

Although the traditional approach of comparing analytical results for stored samples with those for freshly prepared samples has been referred to in this guidance, other statistical approaches based on confidence limits are also available

for the development of SOPs for evaluation of an analyte's stability in a biological matrix (Timm 1985). Whatever approach is used, the SOPs should clearly describe the statistical method and rules employed. Additional validation may include investigation of samples from dosed subjects.

F. Acceptance Criteria

An analytical method is considered fully validated when it meets the following criteria:

Precision: The between-batch CVs for low, medium, and high concentrations should be ≤15%, and ≤20% for the LOQ QC, using a minimum of three batches.

Accuracy: The between-batch mean value should be within ±15% of the nominal value at low, medium, and high QC concentrations and should not deviate by more than ±20% at the LOQ.

Sensitivity: The lowest standard should be accepted as the limit of quantitation of the method if the between-batch CV at the LOQ QC is ≤20%.

Specificity: The responses of interfering peaks at the retention time of the analyte should be less than 20% of the response of an LOQ standard. Responses of interfering peaks at the retention time of the internal standard should be ≤5% of the response of the concentration of the internal standard to be used in studies.

Stability: Long-term, short-term, freeze and thaw, stock solution, and autosampler stability data should meet the criteria specified in the SOP.

V. IN-STUDY VALIDATION

Assays of all samples of an analyte in a biological matrix should be completed within the time period for which stability data are available. In general, analysis of biological samples can be done with a single determination without duplicate or replicate analysis if the assay method has acceptable variability as defined by validation data. This is true for procedures where precision and accuracy variabilities routinely fall within acceptable tolerance limits. For a difficult procedure with a labile analyte, where high precision and accuracy specifications may be difficult to achieve, duplicate or even triplicate analyses may be recommended for better estimate of analyte.

A calibration curve should be generated for each analyte to assay samples in each analytical run and it should be used to calculate the concentration of the analyte in the unknown samples in the run. The spiked samples may contain more than one analyte. An analytical run could consist of either all the processed samples to be analyzed as one batch or a batch composed of processed unknown samples of one or more volunteers in a study, QC samples, and calibration standards. The calibration (standard) curve should cover the expected unknown sample concentration range in addition to a calibrator sample at LOQ. Estimation of concentration in unknown samples by extrapolation of standard curves below LOQ or above the highest standard is not recommended. Instead, the standard curve should be redefined or samples with higher concentration should be diluted and assayed (Shah 1992). All study samples from a subject should be analyzed in a single run.

Once the analytical method has been validated for routine use, its accuracy and precision should be monitored regularly to ensure that the method continues to work satisfactorily. To achieve this objective, a number of separately prepared QC samples should be analyzed with processed test samples at intervals based on the total number of samples. The QC samples in duplicate at three concentrations (one near the LOQ (i.e., $\leq$3 x LOQ), one in midrange, and one close to the high end of the range) should be incorporated in each assay run. The results of the QC samples provide the basis of accepting or rejecting the run. At least four of the six QC samples should be within ±20% of their respective nominal value. Two of the six QC samples may be outside the ±20% of their respective nominal value, but not both at the same concentration (Shah 1992, Brooks 1985, Buick 1990, Mehta 1989, Ayers 1981).

VI. DOCUMENTATION

The validity of an analytical method should be established and verified by laboratory studies. Documentation of successful completion of such studies should be provided in the assay validation report. Protocols that define a set of specific directions that must be followed are important if the analytical results are useful for a given purpose.

General and specific SOPs and good record keeping are essential parts of a validated analytical method. The analytical protocols and SOPs should be signed and dated by the laboratory director and updated regularly. The SOP should state situations under which reassay of samples is permitted. Reassays should be done in triplicate.

The pre-study validation experiments, the data generated from them, and the assay quality control data should be recorded in a bound laboratory notebook. The entries should be signed by the chemist and witnessed by the laboratory supervisor. All records should be available for data audit and inspection.

Documentation for pre-study validation should include:

- A description of the analytical method
- A description of stability studies and supporting data
- A description of experiments conducted to determine accuracy, precision, recovery, specificity, linearity, limit of quantitation, and relevant data obtained from these studies
- Tables of intra- and inter-day precision and accuracy
- Evidence of purity of drug standards, metabolites, and internal standards used in validation experiments
- Deviations from SOP, if any, and justification for deviation

Documentation for in-study validation should include:

- Calibration curves used in analyzing samples and intra-day accuracy and precision data
- Information on inter-day values of QC samples and data on inter-day accuracy and precision from calibration curves and QC samples used for accepting the analytical run
- A protocol for reassay of samples that describes the reasons for reassay and acceptance criteria for reassayed samples
- Reasons for missing samples
- Acceptance criteria for reported values when all unknown samples are assayed in duplicate
- Deviations from the protocol or SOP, with reasons and justifications for the deviations

Documentation for submission to the Agency should include:

- Pre-study validation data
- Calibration curves, equations, and weighting factors used, if any
- In-study validation data

- Complete serial chromatograms of 20% of subjects, with standards and QC samples

- All SOPs, raw data, calculations of concentration, and reassay sample sets

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